

Production of Citric and Oxalic Acids and Solubilization of Calcium Phosphate by *Penicillium bilaii*

JAMES E. CUNNINGHAM* AND CATHY KUIACK

Philom Bios, Inc., 318-111-Research Drive, Saskatoon, Saskatchewan, Canada S7N 3R3

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An isolate of *Penicillium bilaii* previously reported to solubilize mineral phosphates and enhance plant uptake of phosphate was studied. Using agar media with calcium phosphate and the pH indicator alizarin red S, the influence of the medium composition on phosphate solubility and medium acidification was recorded. The major acidic metabolites produced by *P. bilaii* in a sucrose nitrate liquid medium were found to be oxalic acid and citric acid. Citric acid production was promoted under nitrogen-limited conditions, while oxalic acid production was promoted under carbon-limited conditions. Citric acid was produced in both growth and stationary phases, but oxalic acid production occurred only in stationary phase. When submerged cultures which normally produce acid were induced to sporulate, the culture medium shifted toward alkaline rather than acid reaction with growth.

Microbial solubilization of phosphate minerals has been studied as a means of removing phosphate contaminants from iron ore (22) and as an alternative process for producing soluble phosphates from fluorapatite (21). The most intensively studied aspect of microbial phosphate solubilization has been the provision of phosphate for plant uptake by the solubilization of phosphates in rhizosphere environments (1, 2, 9, 12, 13, 17, 18, 20, 30).

Phosphate-solubilizing soil and rhizosphere microorganisms have been distinguished by their relative abilities to dissolve calcium phosphate and apatites in pure culture (10, 30) and in association with plant roots (11). This activity was frequently attributed to organic acid and chelating metabolites (1, 2, 17, 20). However, identification of the metabolites involved has been done in only a minority of the reported investigations (9, 12, 22). Duff et al. (9) identified 2-ketogluconic acid as a major metabolite of various root region bacteria which solubilize mineral phosphates. Phosphate solubilization and the production of 2-ketogluconic acid by *Rhizobium leguminosarum* have also been reported (12). Oxalic acid and various isomers of itaconic acid were reported by Parks et al. (22) to account for the solubilization of apatites by an unidentified, *Penicillium*-like fungus.

Kucey (17) demonstrated increased phosphorus uptake by wheat and beans inoculated with a strain of *Penicillium bilaii* that was previously isolated from soil. The phosphate-solubilizing ability of the *P. bilaii* isolate was determined by the dissolution of precipitated calcium phosphate in an agar medium (16). The rate of phosphate release from rock phosphate in liquid cultures of this fungus was examined by Asea et al. (2), but the identities of the acidic or chelating metabolites involved were not reported. A commercial formulation of *P. bilaii* spores for use on wheat to increase the availability of soluble phosphate for plant uptake has been registered in Canada.

The present investigation was undertaken to identify metabolites produced or other changes occurring in *P. bilaii* cultures that could account for the increased solubility of calcium phosphate observed in agar plates and liquid media. The influence of nutritional and other conditions on the

factors influencing phosphate solubilization were also examined to gain insight into the relevance and interpretation of laboratory assays for microbial phosphate solubilization.

MATERIALS AND METHODS

Microorganism. The organism used in this study was *P. bilaii* Chalabuda, obtained from R. Kucey, Agriculture Canada Research Station, Lethbridge, Alberta, Canada. This isolate has been deposited at the American Type Culture Collection as strain ATCC 20851. The name *P. bilaii* used previously in studies with this isolate (2, 7, 16, 17) has been corrected to *P. bilaii* (24). Conidia were stored with silica gel at -20°C (27). A working silica gel stock was held at 4°C for generation of agar plate cultures (potato dextrose agar [PDA]; Difco) at 2-week intervals. These cultures served as primary inocula for experimental work.

Culturing procedures. In experiments on medium composition, dilute agar spore suspensions (24) were used to inoculate agar plates at single central points. Inocula for liquid culture experiments were prepared by homogenizing approximately 1 cm^2 of mycelium taken from the margin of a PDA plate colony with 50 ml of liquid medium. These cultures were incubated in 125-ml foam-plugged (Dispo) Erlenmeyer flasks at 20°C with gyratory shaking at 200 rpm. After a 4-day incubation, the cultures were rehomogenized and used to inoculate fresh media (in 500-ml Erlenmeyer flasks) with inoculum loads of 5% (vol/vol). Medium composition varied for experimental purposes as given below. The trace elements added to both liquid and solid media, where indicated, were (in milligrams per liter) NaMoO_4 (0.2), H_3BO_4 (2), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.2), FeCl_3 (1.0), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.2), and ZnCl_2 (2.8).

Solid-medium experiments. The agar media used to examine acid production and phosphate solubilization were prepared in 100-ml lots with 0.2 g of CaHPO_4 powder, 1 ml of 1% (wt/vol) alizarin red S (Sigma), or both added to the molten medium (50°C) before plates were poured. Five media were used: PDA (Difco), soil sucrose agar, sucrose nitrate agar, sucrose ammonium agar, and sucrose tryptone agar. All sucrose media contained 20 g of sucrose and 20 g of agar per liter. The soil sucrose agar was prepared from an aqueous extract prepared by mixing garden soil with an equal volume

* Corresponding author.

of water and straining the mixture through cheesecloth. The sucrose nitrate and sucrose ammonium media each contained 5 g of KH_2PO_4 and 1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per liter, trace elements (as listed above), and either 2 g of NaNO_3 or 2 g of NH_4Cl per liter, respectively. The pHs of the sucrose nitrate and sucrose ammonium media were adjusted to pH 6.0 with NaOH. The sucrose tryptone medium contained tryptone (Bacto; 5 g/liter) and trace elements, and no pH adjustment was made.

The effect of these nutrient compositions was recorded by measuring the diameter of the zone of CaHPO_4 solubilization around colonies. Medium acidification was visualized by the presence of the pH indicator alizarin red S; the diameter of the yellow (acid) zone around colonies was recorded. Measurements were made on quadruplicate center-point-inoculated plates after 7 days of incubation at 25°C.

Liquid-medium experiments. (i) **Effect of nitrate and ammonium on phosphate solubilization and identification of organic acid metabolites produced.** Cultures were grown in media consisting of 20 g of sucrose, 5 g of KH_2PO_4 , and 1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per liter, trace elements, and either 2 g of NH_4Cl or 2 g of NaNO_3 per liter as the sole nitrogen source. Media were adjusted to pH 6.0 with NaOH. The $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was autoclaved separately to prevent precipitate formation and consequent pH change. Fresh media and spent media (mycelium removed by filtration through Whatman no. 1 filter paper) were used for phosphate solubility assays. Replicate cultures harvested at approximately 12-h intervals were assayed for organic acids by thin-layer chromatography (TLC) as described below.

(ii) **Time course of citric acid and oxalic acid production.** The basal medium used to examine citrate and oxalate production consisted of 5 g of KH_2PO_4 and 1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per ml and trace elements, and the pH was adjusted to pH 6.0 with NaOH. Carbon-limited cultures contained 10 g of sucrose and 3 g of NaNO_3 per liter. Nitrogen-limited cultures contained 40 g of sucrose and 0.8 g of NaNO_3 per liter. The carbon-nitrogen balance point was previously determined to occur at approximately 17:1 (wt/wt) sucrose- NaNO_3 by measuring maximum biomass yield with 20 g of sucrose per liter and graded amounts of NaNO_3 . The medium compositions for the C-limited and N-limited cultures were selected to result in equivalent mycelial yields in the two media.

Experiments on the effect of oxygen absorption rate (OAR) were done with the basal medium described above plus 30 g of sucrose and 3 g of NaNO_3 per liter. This medium was slightly C limited and thereby permitted the formation of both citric and oxalic acids, as described below in Results. OARs were controlled by using 75, 150, or 250 ml of total culture in 500-ml Erlenmeyer flasks.

Fermentor cultures. Fermentor cultures were grown in 14-liter Chemap type G vessels with 10-liter working volumes. The inocula were prepared by serially expanding 50-ml inoculum cultures to 150-ml cultures in 500-ml Erlenmeyer flasks as described above. The 150-ml cultures were incubated for 3 days and pooled, and 1.0 liter was used to bring the fermentor culture to a final volume of 10 liters. The shake flask culture medium was 30 g of sucrose, 3 g of NaNO_3 , 1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4 g of KH_2PO_4 , and 1 g of K_2HPO_4 per liter with trace elements. The basal medium for fermentor cultures consisted of 40 g of sucrose, 1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1 g of KH_2PO_4 per liter with trace elements. For vegetative cultures, nitrogen was supplied as NaNO_3 (4 g/liter). Calcium ion was required to induce sporulation of *P. bilaii* in liquid culture; therefore, calcium

and nitrogen were supplied to sporulating cultures in the form of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (4.72 g/liter).

The fermentors were operated at 10 liters min^{-1} aeration, 400 rpm agitation, and 30°C. The culture pH was controlled by automatic addition of H_3PO_4 or NaOH. The vegetative culture was controlled to $\text{pH } 6.0 \pm 0.1$; the sporulating culture was controlled to $\text{pH } 4.5 \pm 0.1$ to circumvent the precipitation of calcium phosphate.

Analytical methods. (i) **Culture characterization.** Fungal growth was determined by vacuum filtering and washing mycelium from 10-ml samples of culture by using Whatman no. 1 filter paper. Samples were oven dried for 24 h at 95°C, cooled in a desiccator, and weighed. Medium pH was determined with a glass electrode, and titratable acidity was determined by titrating a 20-ml sample of filtered medium to pH 7.0 with 20 mM NaOH.

(ii) **Phosphate solubility.** The solubility of calcium phosphate was determined by placing 2.0 g of crystalline CaHPO_4 (Fisher Scientific; reagent grade) in 100 ml of test solution (spent and fresh media) in a 500-ml Erlenmeyer flask. The flasks were placed on a gyratory shaker (1-in. [2.54-cm] stroke) at 100 rpm and 20°C. After 1 h of incubation, the solid phase was separated by passing a decanted sample through a membrane filter (0.22- μm pore size). The filtrate was then assayed for P_i content as described below. Preliminary experiments (24-h time courses of CaHPO_4 dissolution) demonstrated that phosphate concentration reached a constant (equilibrium) value in 1 h of incubation.

The pH and phosphate concentration values at equilibrium were recorded by using test solutions of graded initial pH from pH 3.0 to 5.5. All assay mixtures were brought to 50% medium concentration with water after pH adjustment and before CaHPO_4 addition. In this way, the effect of titratable acidity (determined with fresh medium samples adjusted to graded levels of pH with HCl) could be distinguished from the effects of buffering and chelating by metabolites in spent-medium samples.

(iii) **Chemical determinations.** P_i concentration was determined colorimetrically by using the formation of the molybdovanadophosphoric acid complex as described by Maxwell (19). The A_{460} was determined, and a standard curve was generated with KH_2PO_4 . Residual medium carbohydrate was determined by the phenol-sulfuric acid method of Dubois et al. (8). TLC of organic acids was done by a modification of the method of Vega et al. (33). Spent-medium samples were acidified to pH 2.0, and 5- μl aliquots were spotted on cellulose thin layers (Sigmacell type 100 cellulose, 100- μm thickness, polyester-backed plates). The chromatograms were developed with diethyl ether-formic acid-water (70:20:10), and acids were visualized with 0.045% bromophenol blue in 95% ethanol. Organic acid standards included (R_f) oxalic acid (0.11 to 0.41), citric acid (0.45), L-malic acid (0.55), glycolic acid (0.64), succinic acid (0.78), L-lactic acid (0.86), and formic acid (0.92). A dilution series of citric acid indicated that concentrations of 0.03% could be detected.

Colorimetric determination of citric acid was done by the method of Rebelein (25). Oxalic acid determinations were done by using an enzymatic procedure (Sigma; procedure 590). The OAR was determined by the sulfite reduction method of Corman et al. (6) for various volumes in 500-ml foam-plugged Erlenmeyer flasks incubated at 20°C with gyratory shaking at 200 rpm (1-in. stroke).

The identities of citric acid and oxalic acid in spent culture media were confirmed by high-pressure liquid chromatography (HPLC) analysis. Oxalic acid was precipitated from

TABLE 1. Medium pH and CaHPO₄ solubilization resulting from *P. bilaii* growth on various media

Parameter measured	Medium	Diam (cm) observed for medium amendment ^a		
		CaHPO ₄	Alizarin red	Combined
Colony diam	PDA	2.7 ± 0.1	2.2 ± 0.1	2.4 ± 0.2
	Soil sucrose	2.4 ± 0.1	2.5 ± 0.2	2.5 ± 0.2
	Sucrose ammonium	2.3 ± 0.1	2.3 ± 0.2	2.0 ± 0.2
	Sucrose nitrate	2.4 ± 0.2	1.8 ± 0.1	2.1 ± 0.2
	Sucrose tryptone	2.6 ± 0.2	2.1 ± 0.1	2.3 ± 0.2
Yellow (acid) zone	PDA	—	6.4 ± 0.4	3.9 ± 0.2
	Soil sucrose	—	1.1 ± 0.1	1.9 ± 0.2
	Sucrose nitrate	—	2.1 ± 0.1	2.7 ± 0.3
	Sucrose ammonium	—	3.2 ± 0.2	2.8 ± 0.1
	Sucrose tryptone	—	4.4 ± 0.4	3.2 ± 0.3 ^b
CaHPO ₄ -solubilized zone	PDA	3.0 ± 0.1	—	2.7 ± 0.1
	Soil sucrose	1.4 ± 0.1	—	1.3 ± 0.1
	Sucrose nitrate	—	—	—
	Sucrose ammonium	3.0 ± 0.2	—	2.5 ± 0.1
	Sucrose tryptone	3.3 ± 0.1	—	3.5 ± 0.3

^a Each value is the mean ± standard deviation of quadruplicate plates. —, zone is smaller than the colony and therefore not clearly visible.

^b Measurement may have been in error because of pronounced yellow pigment diffusing from the colony.

culture media by adjusting the medium to pH 4.0 (with HCl) and adding 0.2 volumes of 1% (wt/vol) CaCl₂ solution. Calcium oxalate precipitate was collected by centrifugation, washed twice, and dissolved in 1 N HCl for HPLC analysis. To isolate citric acid, spent medium was first adjusted to pH 10.0 (with NaOH) and CaCl₂ was added to precipitate phosphate and oxalate from the sample. Citric acid was then prepared from the supernatant as described for the colorimetric assay and analyzed by HPLC. The concentrations of citrate and oxalate determined by HPLC analysis indicated that there were no interfering substances detected by the colorimetric and enzyme assays applied to the medium samples.

The HPLC system consisted of a Polypore H anion-exchange column (BrownLee Labs; 4.6 mm by 22 cm), a spectrophysics SP8700XR pump, a Kratos Spectroflow 773 UV detector set at 210 nm, and a Hewlett-Packard 3392A integrator. The solvent was 0.01 N H₂SO₄ run at 0.25 ml/min.

RESULTS

Substrate composition and phosphate solubilization. (i) **Phosphate solubilization in agar media.** The data in Table 1 show the influence of medium composition on phosphate solubilization results obtained by the precipitated-phosphate agar technique (11, 30). The production of acid and its diffusion into the media were recorded from media with alizarin red S present and no CaHPO₄. It was evident that on the sucrose nitrate and sucrose ammonium media, which had equivalent buffering capacities, more acidity was produced by growth on ammonium. On media with less buffering capacity than the sucrose nitrate or sucrose ammonium media (PDA and sucrose tryptone), the diameters of the low-pH zones were much greater. On media with CaHPO₄ present and no alizarin red S, the diameters of CaHPO₄-solubilized zones reflected the difference in acid production between sucrose nitrate and sucrose ammonium media.

Plates were also prepared with both alizarin red S and CaHPO₄ present to determine if the presence of alizarin red S influenced the factors causing phosphate solubilization. Analysis of variance of the colony diameters on plates with CaHPO₄ with and without alizarin red S did show a statisti-

cally significant reduction in colony diameter (least significant difference 0.37 cm, *P* = 0.05) due to the presence of alizarin red S. However, the mean difference (all media combined) was only 0.22 cm. The buffering effect of CaHPO₄ is evident when the PDA and sucrose tryptone media with alizarin red S with and without CaHPO₄ are compared.

(ii) **CaHPO₄ solubility in liquid media.** The production of metabolites that affect CaHPO₄ solubility was examined by using sucrose ammonium and sucrose nitrate liquid media. Spent media used for phosphate solubility assays were taken from 72-h-old cultures. These cultures were characterized by recording the time courses of growth and the pHs of replicate cultures (Fig. 1). The difference in pH between the media was thought to be primarily due to the uptake of ammonium ion (26) in the sucrose ammonium medium. Another effect of the medium composition was a slower growth rate in the sucrose ammonium medium than in the sucrose nitrate medium, likely a consequence of the lower pH.

The solubility of CaHPO₄ was determined in solutions of spent and fresh media at initial pHs of 3.0 to 5.5 at intervals of 0.5 pH units. In both types of spent medium, the pH at equilibrium with CaHPO₄ was substantially lower than in the corresponding fresh medium (Fig. 2). One possible explanation for this difference could be buffering of the spent medium by organic acid metabolites. Such buffering would also reduce the protonation of HPO₄²⁻ released from CaHPO₄ dissolution, explaining the lower level of CaHPO₄ solubilized in spent than in fresh sucrose nitrate medium. It was not clear why the equilibrium pH was lower in the spent than the fresh sucrose ammonium medium samples, as organic acids were not detectable by TLC analysis. Also, the equilibrium phosphate concentration was lower in fresh sucrose ammonium medium than in fresh sucrose nitrate medium. These results may have been due to Mg(NH₄)(PO₄) formation, but this possibility was not investigated.

No reduction of phosphate concentration due to assimilation of medium phosphate by *P. bilaii* could be detected within the error limits of the assay. The amount of phosphate in the medium was greatly in excess of that required for growth (3). The presence of citric acid and oxalic acid was indicated in the sucrose nitrate medium cultures by TLC

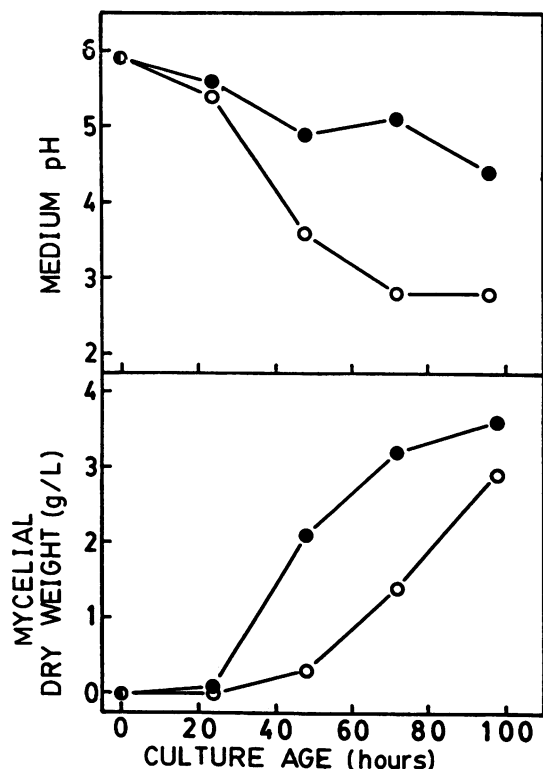


FIG. 1. Time courses of growth and pH in nitrate and ammonium media. *P. bilaii* was grown in liquid media with either sodium nitrate (●) or ammonium chloride (○) as the sole nitrogen source. The results illustrate the increased medium acidity due to ammonium incorporation. Each point represents one independent culture.

analysis. TLC analysis of the sucrose ammonium medium cultures did not detect the presence of organic acid metabolites.

Effect of culture conditions on oxalic acid and citric acid production, titratable acidity, and medium pH. (i) **Carbon-nitrogen balance.** The time courses of growth, pH, and titratable acidity under carbon-limited and nitrogen-limited conditions are shown in Fig. 3A. The time courses of citric acid and oxalic acid in these cultures were also recorded (Fig. 3B). The time courses of growth under carbon-limited and nitrogen-limited conditions were very similar, the maximum mycelial yield in both cultures being approximately 4 g/liter. Stationary phase began at approximately 120 h.

Under nitrogen limitation, the titratable acidity increased throughout growth phase and stationary phase and was 50 mM greater than in the carbon-limited cultures at the end of the incubation period. Accordingly, the final pH of the nitrogen-limited cultures was much lower than that of the carbon-limited cultures. However, the oxalic acid and citric acid analyses only partially explain these observations.

Citric acid was produced during growth phase under both carbon-limited and nitrogen-limited conditions. Under carbon limitation, the citric acid present was partially consumed at the end of growth phase (accompanied by a corresponding pH trend). Under the nitrogen-limited condition, production continued throughout stationary phase.

Very little oxalic acid was produced under nitrogen-limited condition. Oxalic acid accumulated during stationary phase in the carbon-limited cultures. This was not reflected in the titratable acidity of the carbon-limited cultures be-

cause the medium pH remained well above the pK_{a2} of oxalic acid.

(ii) **Oxygen availability.** The production of oxalic acid and citric acid was examined under three conditions of oxygen availability which were adjusted by altering the volume of culture incubated in 500-ml Erlenmeyer flasks. For 75-, 150-, and 250-ml volumes, the OARs were 0.123, 0.073, and 0.025 mmol of O_2 liter⁻¹ min⁻¹, respectively. The time courses of growth, pH, and titratable acidity under the three conditions are shown in Fig. 4A. The corresponding time courses of citric acid and oxalic acid concentrations are shown in Fig. 4B.

The culture growth rate was slightly reduced when OAR was reduced, and a slight decrease in maximum mycelial yield occurred under the lowest OAR. The time courses of oxalic acid and citric acid again show that titratable acidity parallels citric acid production, as noted above. In this experiment, it was found that the production of citric acid was promoted by high oxygen availability. With the high and middle OARs, oxalic acid production occurred at the end of the growth phase, concomitant with citric acid consumption. This was consistent with the carbon-limited condition in the preceding experiment, although the cultures in the OAR experiment were closer to the carbon-nitrogen balance point.

Pigmentation was markedly altered by OAR. With high, middle, and low OARs, the cultures were bright yellow, red-brown, and white, respectively. The pigmentation of the cultures was due to soluble pigments, the white culture having no pigmentation. It was also observed that red was pH sensitive (the cultures becoming yellow when acidified).

(iii) **Sporulation and acid production.** Sporulation in liquid cultures of *P. bilaii* occurs only when the mycelium is exposed to high concentrations of calcium ion, as described by Ugalde and Pitt (32) for *Penicillium cyclopium*. In the present work, it was found that differences in metabolism between sporulating and vegetative cultures resulted in the cultures becoming alkaline and acidic, respectively. This effect was demonstrated by recording the consumption of acid and base in pH-controlled fermentor cultures.

Under nonsporulating conditions (Fig. 5A), the acidification of the medium was evidenced by significant base uptake (approximately 10 mmol/g [dry weight] of mycelial increase). The culture remained vegetative throughout the incubation period. A sporulating culture was similarly examined, as shown in Fig. 5B. In addition to growth and acid and base uptake, spore concentration was monitored as a record of the sporulation process. Under the sporulating condition, a net uptake of acid occurred at a rate of approximately 2.5 mmol of H_3PO_4 per g (dry weight) of mycelial increase. The concomitant uptake of acid and base at the early time points in Fig. 5B resulted from overcompensation by the controller during the period of rapid acid uptake.

DISCUSSION

Observations of agar media (Table 1) demonstrated the influence of substrate composition on the diameter of the zone of calcium phosphate solubilization. Both the nutrient composition and the buffering capacity of the media influenced the results. The increased diffusion of acid into the medium due to ammonium utilization, visualized by the presence of alizarin red S, was also reflected in the diameter of the zone of calcium phosphate solubilization (comparison of sucrose nitrate and sucrose ammonium media). The production of acid and solubilization of calcium phosphate

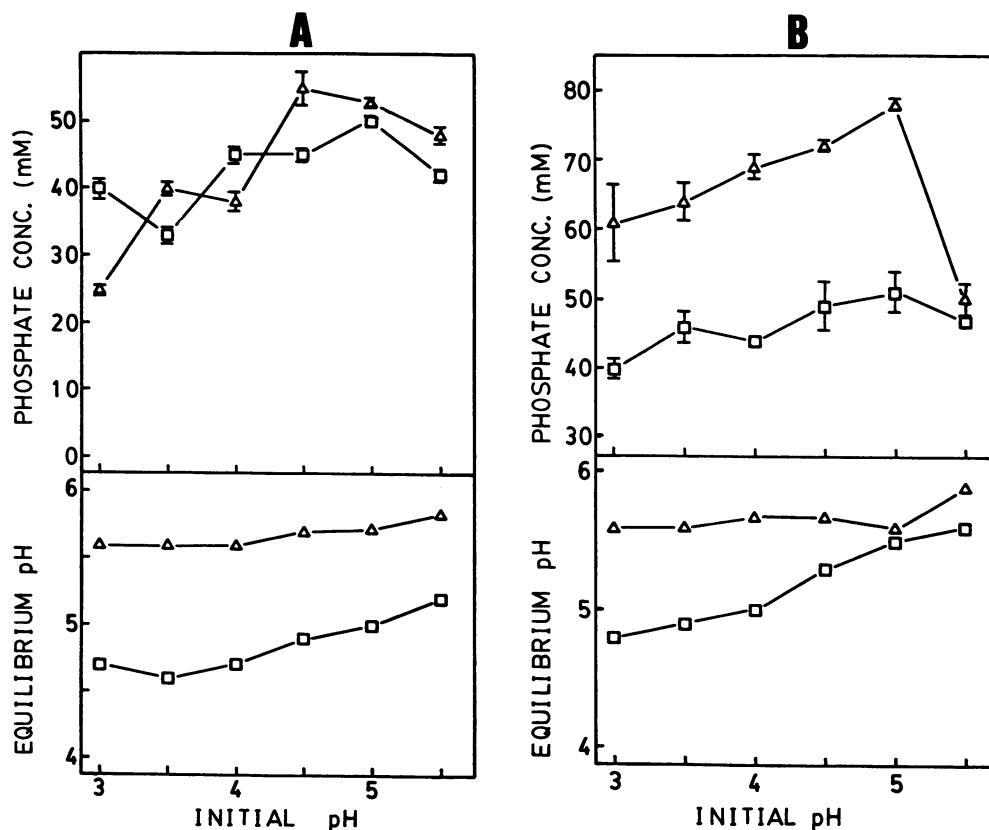


FIG. 2. Determination of calcium phosphate solubility and equilibrium pH in ammonium medium (A) and nitrate medium (B). Samples of spent (\square) and fresh (Δ) media were diluted to 50% of the original concentration and adjusted to graded levels of pH with HCl. The concentration of phosphate was determined after equilibration with $\text{CaHPO}_4(\text{s})$. Bars represent standard deviations. Each point is the mean of duplicate solutions.

were high on complex media (PDA and sucrose tryptone agar). The hydrolysis of peptides and deamination of amino acids (the sole N source) for nitrogen assimilation in the sucrose tryptone medium may have been significant sources of acidity in these cultures.

One implication of these observations is that phosphate solubilization results of this type (10, 13, 16, 30), especially with a limited number of media, should be interpreted cautiously. Since the acid production and metabolite responses vary because of differences in metabolism between organisms and since the response of a single organism varies with substrate, categorizing organisms as phosphate solubilizing and nonsolubilizing becomes difficult. The solubilization activity may therefore be truly meaningful only in the context of the microbes' environments.

Microbial phosphate solubilization on plant roots has been visualized in situ by using an agar contact method (11). An agar contact method with a pH indicator (bromocresol purple) has also been developed for the detection of zones of acidification in the rhizosphere (23) and could be a valuable tool for studying phosphate-solubilizing microorganisms. In situ observations will be needed to fully describe the mechanism of *P. bilaii*-mediated enhancement of phosphate uptake by plants. The present finding that *P. bilaii* synthesizes substantial quantities of citric and oxalic acids provides additional aspects that could be analyzed in situ. Such investigations would also have to distinguish between phosphate released from inorganic sources and that released from

organic compounds such as phospholipids (10, 29). However, separating the effect of phosphate availability from other possible effects of rhizosphere colonization, such as the production of plant growth-regulating substances (4), will be difficult to control experimentally.

In the present study, the equilibrium condition of CaHPO_4 dissolution in cell-free culture media was examined. The purpose was to detect the presence of factors, such as organic acids, that affect the solubility of phosphate in the presence of calcium ion. This approach (the use of cell-free spent media) was also used by Parks et al. (22) in the detection of phosphate solubilization due to oxalic acid and itaconic acid production by a fungal culture.

The equilibrium pH of assay mixtures containing spent media was a dependent variable affected by the presence of indeterminate amounts of organic acid metabolites. The relationship between initial pH, equilibrium pH, and phosphate concentration is further complicated by the formation of complexes with calcium and magnesium (5) in the assay mixtures. Therefore, the solubility of CaHPO_4 was recorded in samples of media adjusted to graded levels of initial pH.

Since the forward rates for the formation of less-soluble calcium phosphate compounds, such as apatite, are very slow (31), the concentrations of phosphate ion observed were regarded as representing the equilibrium condition with respect to CaHPO_4 solubility. The ability to detect chelation was demonstrated by determining the concentration of phosphate ion in 0.037 M KH_2PO_4 solutions after equilibration

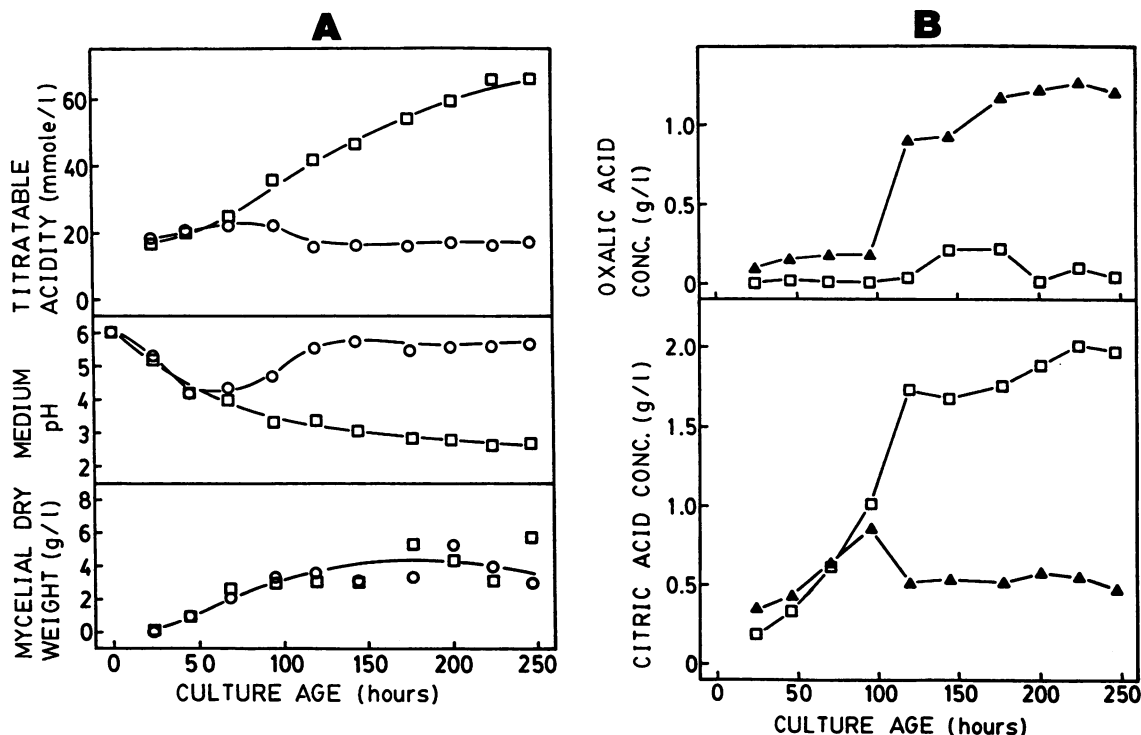


FIG. 3. Time courses of growth, pH, titratable acidity, oxalic acid concentration, and citric acid concentration under carbon limitation and nitrogen limitation. (A) C limitation (○) and N limitation (□); (B) C limitation (▲) and N limitation (□).

with CaHPO_4 with and without 0.026 M citric acid. Both solutions were adjusted to pH 4.5 with NaOH or HCl prior to addition of CaHPO_4 . In the presence of citrate, the equilibrium concentration of phosphate was 0.027 M greater than that of the control. The difference was presumed to be

primarily due to chelation, as the equilibrium pH values were the same (pH 4.45).

In other liquid culture studies (1, 2, 12, 16, 21), insoluble phosphates, including tricalcium phosphate, hydroxyapatite, fluorapatite, and rock phosphate from various sources, were

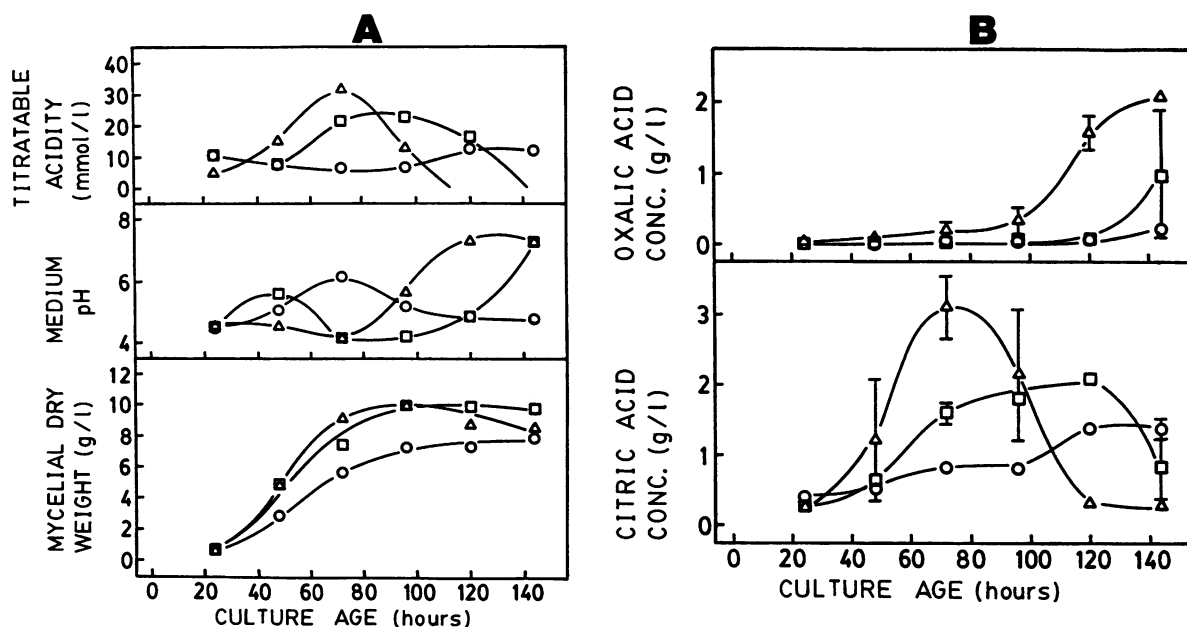


FIG. 4. Time courses of growth, pH, titratable acidity, oxalic acid concentration, and citric acid concentration under three conditions of oxygen availability. The three OAR conditions examined were 0.123 (Δ), 0.073 (□), and 0.025 (○) mmol of O_2 liter $^{-1}$ min $^{-1}$. Each point is the mean of two cultures. Error bars in panel B indicate standard deviations.

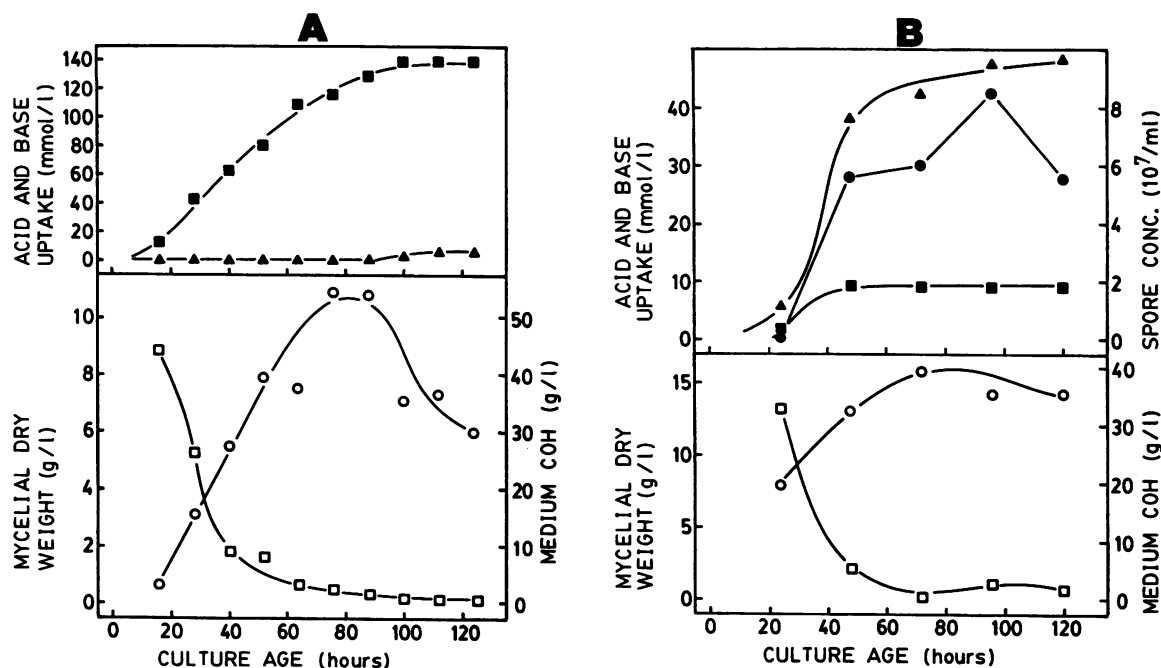


FIG. 5. Effect of sporulation on acid production. The time courses of mycelial dry weight (○), residual medium carbohydrate (□), acid uptake (▲), base uptake (■), and spore concentration (●) were recorded for a vegetative culture (A) and a sporulating culture (B).

placed directly into the growth medium with the test organism. Thus, the time courses of phosphate ion concentration observed in these cultures reflected the organisms growth rate, the rate and amount of acid produced by the organism, and the kinetics of dissolution and formation of the precipitates and complexes involved.

The use of rock phosphate to detect phosphate solubilization was also examined in the course of this study by replacing CaHPO_4 with Florida rock phosphate in the standard assay procedure. In contrast to the rapid attainment of equilibrium by CaHPO_4 , rock phosphate produced fluctuating phosphate concentrations over the 24 h of observation. While there were several possible explanations for this observation, the solubility of rock phosphate was not examined further, as this substance was clearly not suited for the rapid detection of phosphate-solubilizing metabolites. Purified apatitic materials were not evaluated.

Synthetic media containing sucrose and inorganic nitrogen sources were used to identify phosphate-solubilizing metabolites with minimal interference from medium components. The components of the media were similar to those in studies done elsewhere with the same organism (2). The initial tests gave evidence of organic acids (causing buffering) and increased medium acidity due to ammonium assimilation (Fig. 2 and 3). TLC analyses of cultures grown in these media showed that citric acid was produced in the nitrate medium but was not detectable in the ammonium medium. It also appeared that oxalic acid was present in older cultures, although it was obscured by medium components and poor resolution with the solvent system used. Traces of other acidic metabolites were present; however, these did not closely match the standards and were not positively identified. HPLC data confirmed the identities of oxalic and citric acids.

The production of oxalic acid by a hyphomycete (*Aspergillus niger*) was reported as early as 1891 (28), and the

effect of the carbon-nitrogen ratio of the medium on citric acid and oxalic acid production was described in 1917 (28). Under conditions of nitrogen limitation, a high conversion of carbohydrate to citric acid is known to occur with some fungi (14). Yields of citric acid as high as 90% can be obtained from sucrose by *A. niger* fermentations (15). The production of citric acid and oxalic acid by *P. bilaii* was consistent with these observations.

Of the titratable acidity values recorded in Fig. 3A, 12 mM was the neutralization point of medium phosphate (the medium was initially pH 6.0, and titratable acidity was determined by titrating to pH 7.0). In the nitrogen-limited cultures, calculations showed that the major organic acid metabolite (citric acid) accounted for only 59% of the titratable acidity produced by *P. bilaii*. In carbon-limited cultures, the final amount of titratable acidity produced by *P. bilaii* was only 4 mM, despite the presence of 9 mM oxalic acid. This amount of oxalic acid would have contributed 18 mM titratable acidity had it not been neutralized by other changes in the medium.

It is evident from these data that the net change in titratable acidity reflected the overall conversion of sucrose and nitrate to *P. bilaii* biomass and metabolites. The accumulation of specific metabolites and the net change in titratable acidity depend on the metabolic pathways active under the different incubation conditions. This was particularly evident in the sporulating culture (Fig. 5B), in which *P. bilaii* caused a net reduction of titratable acidity in the medium. This sporulating culture was shown to become alkaline at a rate of approximately 3 mmol/g (dry weight) of mycelial increase, whereas a vegetative culture produced acid at a rate of 10 mmol/g (dry weight) of mycelial increase (Fig. 5). In addition, the levels of citric acid and oxalic acid were shown to be modulated by oxygen availability (Fig. 4).

Asea et al. (2) reported values for the effect of *P. bilaii* on total P uptake by plants. Using two wheat plants per pot (1

kg) of soil, total P uptake by the plants was typically in the range of 0.23 mmol. The utilization of rock phosphate and total P uptake was increased (by about 20 to 25%) by the presence of *P. bilaii* (2). The uptake of ammonium ion by *P. bilaii* in liquid cultures (data not shown) was approximately 4.7 mmol/g (dry weight) of mycelial growth in sucrose ammonium medium. This indicates that approximately 4.7 mmol of acid may be produced per g of mycelial growth because of ammonium assimilation alone (26). The amounts of citric acid and oxalic acid produced in liquid cultures were in the range of 4 to 5 mmol of acid equivalents per g (dry weight) of mycelial growth.

In summary, it was evident that citric acid, oxalic acid, and acidity resulting from ammonium incorporation are significant components of the laboratory culture observations of phosphate solubilization by *P. bilaii*. However, these factors which account for the phosphate solubilization effect are highly dependent on the nutrition and physiological status (e.g., sporulation) of the culture and the physical conditions (e.g., aeration) under which the culture is grown. Further investigation will need to focus on the action of *P. bilaii* under *in situ* conditions in order to fully describe its effect in the rhizosphere.

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